Tissue Residues and Metabolism of Avilamycin in Swine and Rats

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[^{14}C]Avilamycin was fed to growing swine at a level of 60–80 ppm (1.5–2 times the recommended use level), and tissues were assayed for radioactivity (RA). At a practical zero withdrawal swine fed 60 ppm of uniformly labeled (U-^{14}C) avilamycin for 14 days had RA residues of 0.14, 0.66, 0.34, and 0.55 ppm in muscle, liver, kidney, and fat, respectively. Swine fed 80 ppm of [^{14}C]avilamycin labeled in the dichloroisoeverninic acid portion had residues 3–5 times lower, indicative that most of the residue was derived from the oligosaccharide portion of avilamycin. The primary metabolite in liver and feces was flambic acid. Most of the RA in fat from swine fed [U-^{14}C]avilamycin was in the fatty acids. [^{14}C]-Avilamycin was excreted rapidly and nearly quantitatively by swine, with 5% of the dose in the urine and the remainder in feces. The excretion pattern and metabolic profile of [^{14}C]avilamycin in the rat were similar to swine.

INTRODUCTION

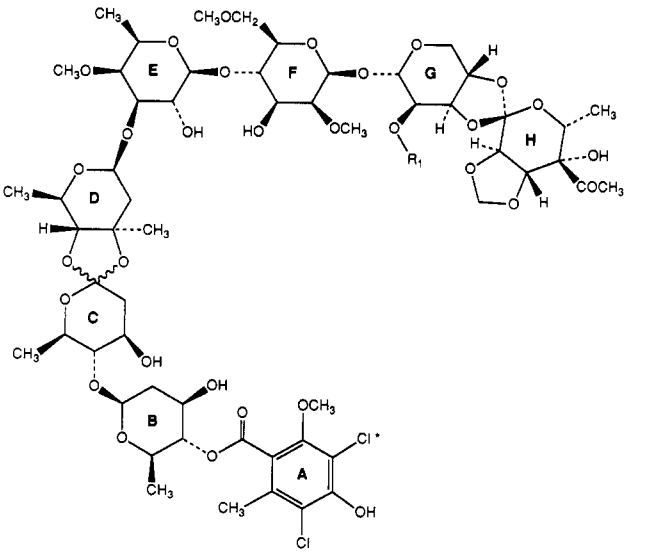
Avilamycin is an antibiotic of the orthosomycin family that consists of a six-member oligosaccharide, dichloroisoeverninic acid (DCIEA), and methyl eurekanate. The avilamycin complex is produced by *Streptomyces viridochromogenes* and consists of factor A as the major component with several other minor components. The structures have been published (Mertz et al., 1986) and the primary components are shown in Figure 1. Avilamycin, when fed to swine, causes an increase in rate of gain and efficiency of feed utilization (Jones et al., 1987a,b; Watkins et al., 1987).

MATERIALS AND METHODS

Labeled Compound. [^{14}C]Avilamycin was prepared by fermentation using two different substrates. [^{14}C]Glucose substrate produced relatively uniformly labeled avilamycin. Hydrolysis and characterization of this material demonstrated that seven of the eight rings (all except ring D, Figure 1) contained radioactivity. Ring D is presumed to be labeled also, but the characterization was terminated short of confirmation of that labeling. A second substrate, [^{14}C]diethylmalonate, produced avilamycin in which approximately 85% of the label was associated with the DCIEA ring and the remainder was in the rest of the molecule. The two labeled materials were called [U-^{14}C]-avilamycin and [DCIEA-^{14}C]avilamycin, respectively. [^{14}C]-Avilamycin dosing materials were purified to 98% or greater radiochemical purity before use.

Animal Feeding, Dosing, and Sample Collection. Balance-excretion experiment SW-1 was conducted to determine the rate, route, and extent of excretion of [^{14}C]avilamycin. Two female pigs weighing approximately 40 kg were housed individually in metabolism cages and fed a standard grower ration containing 60 ppm of unlabeled avilamycin. On the day of dosing, the pigs were given a 120-mg dose of [U-^{14}C]avilamycin, 0.25 μCi/mg, mixed into 450 g of ration. After consumption of the dose, they were fed 450 g of unmedicated ration and were maintained on unmedicated ration for the remainder of the experiment. Urine and feces were collected quantitatively daily and frozen.

For the tissue residue studies, crossbred barrows (castrate males) and females weighing 40–50 kg were housed individually in metabolism cages and conditioned on an unmedicated grower-finisher ration. The pigs were then fed twice daily a [^{14}C]-avilamycin-treated ration equivalent to nominal treatment levels of 80 or 60 ppm. The quantity of [^{14}C]avilamycin needed daily was calculated on the basis of feed consumption of 4% of body weight. This dose was administered in a portion of feed medicated at twice the calculated treatment level. The pigs were allowed to consume the treated ration and then were given a portion of unmedicated ration. This regimen was used to ensure that the confined pigs actually consumed the appropriate dose. Treated feed was prepared by dissolving the appropriate quantity of [^{14}C]-avilamycin in 50–60 mL of acetone and pipetting this solution dropwise over the surface of 4 kg of control ration. The acetone was allowed to evaporate, and this "premix" was thoroughly mixed into the remainder of the ration.



Factor	R_1	Other Modifications
A	-CO-CH(CH$_3$)$_2$	
A'	-CO-CH$_2$-CH$_3$	-H replaces -COCH$_3$ on Ring H
B	-COCH$_3$	
E	- H	-CHOH-CH$_3$ replaces -COCH$_3$ on Ring H
F	-CO-CH(CH$_3$)$_2$	-OH replaces -OCH$_3$ and H replaces the Cl marked by * on Ring A
G	-CO-C$_4$H$_9$	
H	-CO-CH(CH$_3$)$_2$	-H replaces the Cl marked by * on Ring A
I	-CO-CH$_2$-CH$_3$	
J	-CO-CH(CH$_3$)$_2$	-OH replaces -OCH$_3$ on Ring F

Figure 1. Structure of avilamycins.

Prior to tissue sampling, the swine were sacrificed with a captive bolt device, exsanguinated as completely as possible, and then washed with a high-pressure hose to remove feed and fecal contamination. Samples of lumbar backfat and muscle (*Longissimus dorsi*) along with the liver and kidneys were then collected.

Experiment SW-2 was conducted to determine the steady-state concentrations of RA in tissues of swine fed 80 ppm of [DCIEA-^{14}C]avilamycin, 0.242 μCi/mg. Nine pigs, five males

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0021-8561/91/1439-0306$02.50/0

and four females, were fed the treated ration. After dosing for 4, 7, or 10 days, groups of three pigs were euthanized at a practical zero withdrawal time of 6 h after the last dose and tissues were taken for assay.

Experiment SW-3 was conducted to determine the rate of decline of residue after withdrawal from treatment. Five pigs were fed the equivalent of 80 ppm of [DCIEA-^{14}C]avilamycin, 0.249 μCi/mg, for 7 days and euthanized at zero withdrawal (6 h), 3 days, or 5 days after the last dose.

Experiment SW-4 was conducted to determine steady-state residues in tissues of swine fed 60 ppm of [U-^{14}C]avilamycin. Six pigs were fed [U-^{14}C]avilamycin, 0.269 μCi/mg, and groups of three pigs were euthanized at zero withdrawal after dosing for 10 and 14 days.

Rats were dosed with [^{14}C]avilamycin to provide samples for characterization of radioactivity in excreta and tissues. In experiment RT-1, three male and three female Sprague-Dawley rats were housed in metabolism cages and dosed once daily for 3 days with [DCIEA-^{14}C]avilamycin, 100 mg/kg of body weight, in acacia suspension. Urine and feces were collected quantitatively, through the 24-h period following the third dose. In experiment RT-2, three male and three female Sprague-Dawley rats were fed a ration containing 550 ppm of [U-^{14}C]avilamycin for 4.5 days. Urine and feces were collected during dosing, and livers were taken from the rats at zero withdrawal.

Sample Preparation and Analysis. Urine and feces were collected daily in the balance-excretion experiments. Feces samples were diluted with an equal weight of water and were homogenized to give a uniform paste. Tissues from residue experiments were ground and frozen until assayed. Five nominal 0.5-g replicates of muscle, liver, and kidney were each digested in 3 mL of NCS solubilizer (Amersham Corp.). After several days, a toluene-based scintillation cocktail was added for liquid scintillation counting (LSC). Five nominal 1-g replicates of fat were melted at approximately 80 °C in a counting vial and then combined with Aquasol (New England Nuclear Corp.) for LSC. Aliquots of urines were combined with a dioxane–Methyl Cellosolve–toluene based cocktail and assayed by LSC. Feces, extracted tissue solids, column packing materials, and feeds were assayed by combustion to $^{14}CO_2$, followed by LSC, as described by Herberg et al. (1978). Samples were counted in a Tri-Carb liquid scintillation counter (Packard Instruments Co.), and count rates were adjusted to 100% efficiency by internal standardization with [^{14}C]toluene.

Tissues from untreated pigs were assayed to provide background data. The limit of detection (LOD) for tissue samples was defined as control counts per minute (CPM) + 3 standard deviations, or + 4 cpm, whichever was greater. In most experiments, recovery of RA was determined by assay of "spiked" control tissues to establish a limit of quantitation (LOQ).

Characterization of Residues in Tissues and Excreta. Feces samples of 20 (swine) or 10 g (rats) were extracted by blending for 15 min in 200 mL of acetone. Each sample was filtered by vacuum filtration, and the filter cake was extracted under reflux for 30 min in 200 mL of 1:1 acetone/water. The sample was filtered and fractions were assayed for RA. Liver samples of 50 (swine) or 5 g (rat) were extracted sequentially by blending in acetone and methanol and then by reflux in 1:1 acetone/water. Solvent volumes were 4 and 20 mL/g for swine and rat samples, respectively.

Column chromatography was performed with an 8 mm i.d. × 120 cm glass column packed with 50 mL of Woelm dry column silica gel (Universal Scientific, Inc., Atlanta, GA). The sample was dried onto 10 mL of the silica gel and packed into the column. A nonlinear gradient was developed in a 190-mL stirred reservoir filled initially with toluene. The elution was performed with 200 mL of each of the following solvents in sequence: 2:1 toluene/ethyl acetate (EA), 100% EA, 8:2 EA/methanol, 8:2:0.1 EA/methanol/acetic acid, 6:4 EA/methanol, 100% methanol. Twenty-milliliter fractions were collected, and aliquots were assayed by LSC.

Thin-layer chromatography (TLC) was performed on 20 × 20 cm silica gel 60 F_{254} plates (E. Merck) developed in solvent I (85:15:4 EA/cyclohexane/methanol), solvent II (95:5:2 EA/methanol/acetic acid), or solvent III (9:1 EA/methanol). Radioactive zones were visualized on TLC plates by autoradiography with X-OMat film (Kodak).

Table I. Excretion of Single Doses of [^{14}C]Avilamycin by Swine

	% of administered dose				
	pig 594		pig 596		
days postdose	urine	feces	urine	feces	mean cumulative
1	2.8	0.1	3.3	0.0	3.1
2	0.7	65.7	0.9	18.2	45.9
3	0.2	24.5	0.3	72.8	94.8
4	0.3	1.6	0.0	2.2	96.8
5	0.1	0.5	0.3	0.5	97.5
6–9	0.0	0.4	0.2	0.4	98.0
total	4.1	92.8	5.0	94.1	
total excreted	96.9		99.1		98.0

Selected samples were assayed for DCIEA by basic hydrolysis of extracts and gas–liquid chromatography (GLC) assay of the DCIEA after methylation with diazomethane. GLC was performed with a Hewlett-Packard Model 5730 instrument equipped with electron capture detector. The column was 3% Carbowax 20M on Chromosorb W-HP, eluted with 10% methane in argon at an oven temperature of 200 °C.

Fat samples were melted in beakers on a hot plate, decanted from connective tissue, cooled, and assayed by LSC. Aliquots were saponified at 55 °C in 5 mL/g 50% aqueous NaOH for 4–6 h, followed by dilution with 2 volumes of water and heating for an additional 40 h. A sample equivalent to 2 g of melted fat was washed into a separator with 120 mL of water and extracted with 2 × 100 mL of $CHCl_3$. Fatty acids were extracted into 100 + 50 + 50 mL of $CHCl_3$ after adjustment to pH 3. Nominal 0.5-g samples of fatty acids were dissolved in 50 mL of absolute ethanol, neutralized with 2 N NaOH, and evaporated to ca. 30 mL to remove traces of water. The *p*-bromophenacyl esters were prepared by reaction with 0.5 g of *p*-bromophenacyl bromide under reflux for 2 h. The reaction mixture was cooled and diluted with 100 mL of hexane, 180 mL of methanol, and 40 mL of water. The hexane was removed after separation of phases, and the aqueous alcohol was extracted additionally with 100 + 50 + 50 mL of hexane. The combined hexanes containing the phenacyl esters were purified by recrystallization from methanol. The progress of purification was monitored by HPLC using a Waters μBondapak C_{18} 4.6 mm × 25 cm column eluted with 95:5 methanol/water with detection at 254 nm.

RESULTS

Excretion of [^{14}C]Avilamycin. The rate, route, and extent of excretion of [^{14}C]avilamycin by swine in experiment SW-1 are shown in Table I. Recovery of the dose averaged 98% for the two pigs. Approximately 93% of the dose was excreted in the feces, and 5% was excreted in the urine. Approximately 95% of the administered dose was excreted within 3 days after dosing. In rat metabolism experiment RT-1, urine contained less than 0.5% of the administered dose, while feces contained 96% (n = 6 animals).

Tissue Radioactivity Concentrations. *Experiment SW-2.* Results from the [DCIEA-^{14}C]avilamycin steady-state study, expressed as avilamycin equivalents in tissues, are shown in Table II. There was no detectable residue of RA in muscle (<0.02 ppm). Liver, kidney, and fat contained 0.2, 0.1, and 0.1 ppm, respectively. RA concentrations achieved steady state in liver and kidney after 7 days of dosing since 10-day concentrations were not significantly higher (one way ANOVA, $P > 0.05$) than 7-day concentrations. There was a suggestion of progressively higher RA in fat with longer dosing times. Liver, kidney, and fat from the three pigs in the 10-day dose group were assayed for parent avilamycin by thin-layer bioautography and for DCIEA by GLC. Kidney and fat contained

Table II. Residues of Radioactivity in Tissues of Pigs Fed 80 ppm of [DCIEA-^{14}C]Avilamycin for 4–10 Days and Euthanized at Zero Withdrawal

animals	dosing interval, days	ppm net radioactivity (mean ± SD)			
		muscle	liver	kidney	fat
2 males, 1 female	4	NDR[a]	0.211 ± 0.029	0.099 ± 0.005	0.057 ± 0.012
1 male, 2 females	7	NDR	0.226 ± 0.035	0.097 ± 0.005	0.080 ± 0.007
2 males, 1 female	10	NDR	0.216 ± 0.028	0.102 ± 0.006	0.121 ± 0.002

[a] No detectable residue at a limit of detection of 0.02 ppm.

Table III. Residues of Radioactivity in Tissues of Pigs Fed 80 ppm of [DCIEA-^{14}C]Avilamycin for 7 Days and Euthanized at Various Withdrawals

animals	withdrawal, days	ppm net radioactivity			
		muscle	liver	kidney	fat
female	0	NDR[a]	0.147	0.077	0.067
male	3	NDR	NDR	0.026	0.044
female		NDR	NDR	0.022	0.062
male	5	NDR	NDR	NDR	0.047
female		NDR	NDR	0.025	0.049

[a] No detectable residue at a limit of detection of 0.02 ppm.

no detectable residue of parent avilamycin (LOQ of 0.05 ppm). Liver contained traces of avilamycin, but concentrations were below 0.05 ppm. Kidney and fat contained no detectable DCIEA (LOQ of 0.1 ppm), while liver contained a mean of 0.126 ppm. Thus, most of the liver RA consisted of residue containing the DCIEA moiety, but very little was parent avilamycin.

Experiment SW-3. Results from the [DCIEA-^{14}C]-avilamycin tissue residue decline study are shown in Table III. The residue pattern at zero withdrawal was consistent with the previous experiment. Residues in liver and kidney declined to near nondetectable concentrations by 3 days. Residue in fat declined more slowly.

Experiment SW-4. Results from the [U-^{14}C]avilamycin steady-state study are shown in Table IV. Muscle, liver, and kidney residues were approximately 0.1, 0.6, and 0.3 ppm, respectively. Residues after 14 days of dosing were not significantly higher than for 10 days. The residues in fat were statistically higher in the 14-day group (0.55 vs 0.26 ppm). Comparison of these data with experiment SW-2 shows that dosing with uniformly labeled avilamycin gave residues 3–5 times higher than when the label was primarily in the DCIEA portion of the molecule.

Characterization of RA in Feces and Urine. Twenty-gram samples of feces from swine fed [DCIEA-^{14}C]avilamycin (experiment SW-2) and swine fed [U-^{14}C]-avilamycin (experiment SW-4) and 10 g of feces from rats fed [U-^{14}C]avilamycin (experiment RT-1) were extracted as described under Materials and Methods. Silica gel column chromatograms were generated for each feces sample on pooled 10% aliquots of the acetone and acetone/water extracts. A comparison of the distribution of RA is shown in Figure 2, and the chromatograms are compared in Figure 3. More than 95% of the RA was extracted from each sample, and the extractable RA gave qualitatively similar radiochromatograms with three major peaks (Figure 3). The distribution of RA and chromatographic profile was quantitatively similar for the [U-^{14}C]avilamycin-treated pigs and rats. The feces from the [DCIEA-^{14}C]-avilamycin-treated pigs yielded most of the residue in the acetone/water extract. The chromatogram from this sample indicated that most of the sample RA was in peak 3.

Pools of fractions from peaks 1–3 were prepared for each feces sample, and these were evaluated by TLC. Results are shown in Figure 4. Peak 1 is the region where parent avilamycin elutes from the silica gel column. TLC of peak 1 samples demonstrated that the peak consisted primarily of two components, avilamycin factor A at an R_f of ca. 0.6 and an unidentified compound at R_f of ca. 0.4. There was good qualitative agreement among all three feces samples. Avilamycin constituted approximately 8% of the fecal RA from the U-^{14}C-treated pig. The unidentified compound had an apparent MW of 1404, 2 mass units greater than that of avilamycin, by negative fast atom bombardment mass spectrometry. It was not characterized further since it was not a major metabolite in tissues of treated swine.

TLC of peak 2 samples demonstrated a qualitative difference between U-^{14}C- and DCIEA-^{14}C-treated animals. Feces fractions from swine and rats treated with [U-^{14}C]-avilamycin contained three metabolites which moved just off the point of application (origin). In contrast, the RA in the sample from the DCIEA-^{14}C-treated swine was primarily polar material which remained at the origin. These results indicate that the three metabolites were derived from the oligosaccharide and/or eurekanate portion of avilamycin.

TLC of peak 3 samples demonstrated that most of the RA was in a single compound. It was relatively polar and required an acidic TLC solvent for mobility. This compound was subsequently determined to be flambic acid (Figure 5). It was relatively unstable and cyclized readily to flambalactone, which has a mobility slightly greater than avilamycin. Quantitation by counting silica gel segments scraped from the plate indicated that these two zones constituted 80% or more of the peak 3 RA for both swine and rats.

Similar column and TLC fractionations were conducted on urines from [U-^{14}C]avilamycin-treated swine and rats. The results indicated the presence of the same primary metabolites as were observed in feces samples.

Characterization of RA in Livers and Kidneys of Swine and Rats. Preliminary characterization was done on liver from [DCIEA-^{14}C]avilamycin treated pigs to determine the residue profile. This work indicated that there was very little parent avilamycin present and that the only major metabolite was a nonpolar compound which was identified as flambalactone. Two minor metabolites were observed by TLC. One had a TLC mobility similar to that of the unidentified peak 1 metabolite from feces, and the other was a polar acidic compound. Further work indicated that the polar acidic compound was flambic acid (Figure 5) and that it was undergoing conversion to flambalactone during sample processing.

Subsequently, fractionation was performed on livers from [U-^{14}C]avilamycin-treated pigs and rats by procedures described under Materials and Methods. Liver samples were extracted with acetone, methanol, and 1:1 acetone/water. The distribution of radioactivity from six individual pigs and a pool of rat livers is shown in Table V. Representative column radiochromatograms from one male and one female pig and the female rat pool are shown in Figure 6.

The liver chromatograms had three distinct features. There were the peak 1, peak 2, and peak 3 regions

Table IV. Residues of Radioactivity in Tissues of Pigs Fed 60 ppm of [U-^{14}C]Avilamycin

animals	dosing interval, days	ppm net radioactivity (mean ± SD) muscle	liver	kidney	fat
2 males, 1 female	10	0.093 ± 0.036	0.554 ± 0.143	0.321 ± 0.083	0.261 ± 0.094
2 males, 1 female	14	0.135 ± 0.031	0.659 ± 0.159	0.340 ± 0.010	0.551 ± 0.034

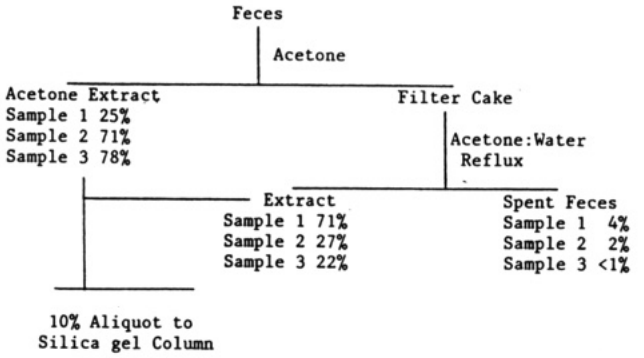

Figure 2. Distribution of radioactivity in feces extracts. Samples 1–3 are [DCIEA-^{14}C]avilamycin-treated pig, [U-^{14}C]avilamycin-treated pig, and [U-^{14}C]avilamycin-treated rat, respectively.

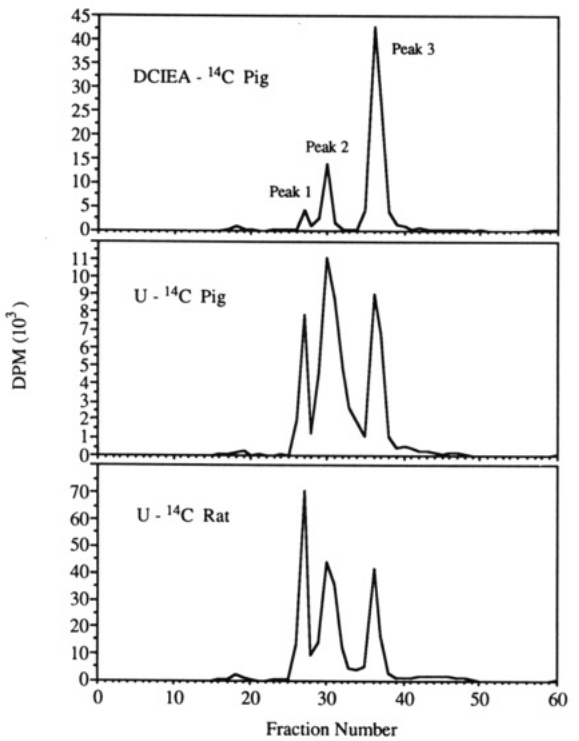

Figure 3. Silica gel column radiochromatograms of feces extracts.

qualitatively similar to the feces samples. There was a nonpolar region in fractions 1–10 and a polar region after peak 3. In both of the swine liver samples the peak 1 RA was less than 0.02 ppm, which demonstrates that very little of the RA was parent avilamycin. This result is consistent with the results from experiment SW-2 in which only traces <0.05 ppm of avilamycin were detectable in livers. The most abundant metabolite was flambic acid (peak 3), which accounted for 6–8% of the liver residue. The identity of flambic acid in liver was confirmed by conversion to methyl flambate (Figure 5) and comparison by HPLC to authentic standard. The nonpolar RA amounted to less than 7% of the total. No specific compounds were identified from this fraction. However, the presence of labeled fatty acids in swine fat as described in the following section suggests that this RA may be in the liver lipids. The greatest amount of RA was in the

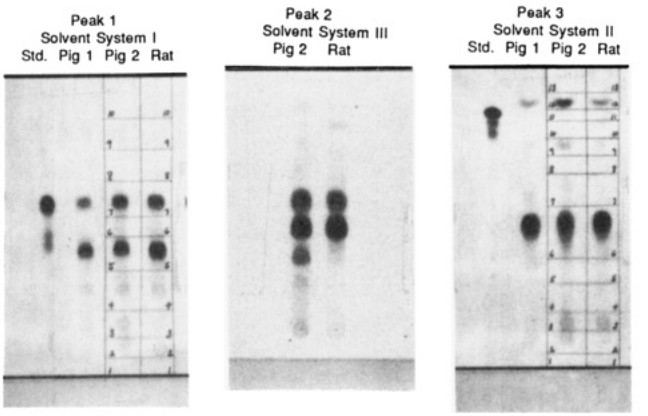

Figure 4. Autoradiograms from silica gel column peaks 1–3. Pig 1, [DCIEA-^{14}C]avilamycin-fed pig; pig 2, [U-^{14}C]avilamycin-fed pig.

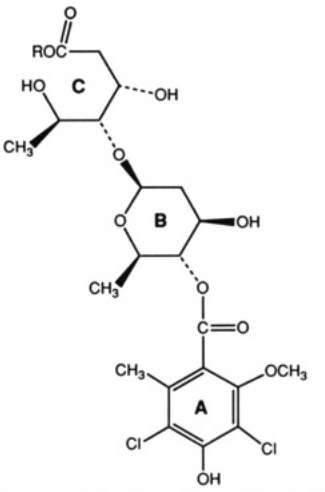

Figure 5. Structures of flambic acid and its derivatives. Flambic acid, R = H; methyl flambate, R = CH_3; flambalactone, ROH removed to form lactone on ring C.

Table V. Distribution of Radioactivity in Extracts of Liver from [U-^{14}C]Avilamycin-Treated Rats and Pigs

fraction	female rat, %	male rat, %	six pigs, % ± SD
acetone	26.8	27.2	32.3 ± 1.9
methanol	36.8	30.5	25.7 ± 1.4
acetone/water	4.1	*a*/	6.7 ± 0.8
spent liver	32.3	42.3	35.0 ± 1.7

[a] Acetone/water extraction was not done on this sample.

polar region of the chromatogram. Unfortunately, this was intractable RA which was distributed among many fractions. The polar column eluate contained too many tissue coextractives for further characterization of residues.

Twenty-gram samples of pooled kidney samples from [U-^{14}C]avilamycin-treated swine were processed similarly to the liver samples. The distribution and chromatographic profile were qualitatively similar to the results presented for liver. There was a slightly higher proportion of the RA present as parent avilamycin in kidney than in liver.

Characterization of Radioactivity in Fat. *p*-Bromophenacyl fatty acid esters were prepared from fat of treated pigs as described under Materials and Methods.

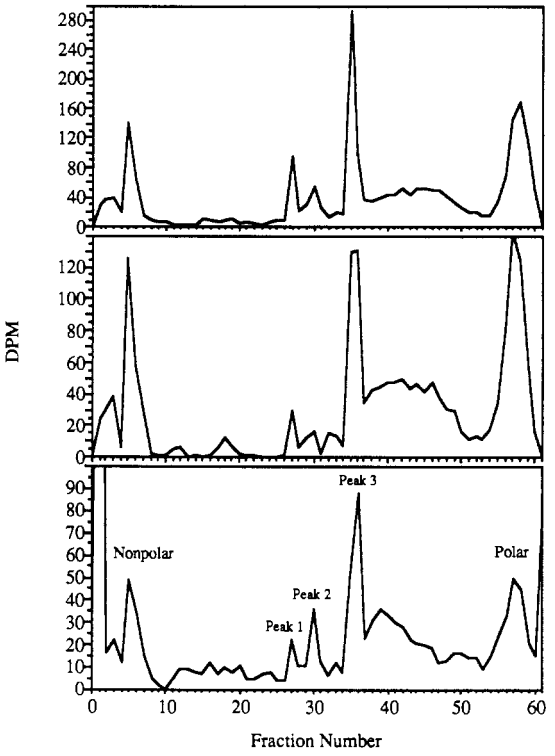

Figure 6. Silica gel column radiochromatograms of liver extracts. Female pig (top); male pig (middle); rat (bottom).

Multiple recrystallizations gave samples with constant specific activity which were almost entirely oleic and stearic acid esters. For three pigs, the disintegrations per minute per gram in melted fat were 475, 386, and 442. The corresponding disintegrations per minute per gram in the fatty acids, after correction for the molecular weight of the unlabeled alcohol, were 564, 517, and 585, respectively. Thus, the specific activity of the fatty acids was higher than that of the original melted fat. Since oleic and stearic acids are the major components of swine fat, these results indicate that most, if not all, of the RA was in the fatty acids.

DISCUSSION

Results from these studies indicate that oral doses of avilamycin are excreted primarily in the feces by swine and rats. Most of the parent avilamycin was metabolized or degraded, since only about 8% of the fecal RA was avilamycin. The primary fecal metabolite was flambic acid. Animals dosed with [U-^{14}C]avilamycin produced three unidentified fecal metabolites that were derived from the oligosaccharide and/or eurekanate moieties.

Mean residues in muscle were all below 0.2 ppm, and residues in other edible tissues were all below 1 ppm. Most of this residue was derived from the oligosaccharide and/or eurekanate portion of avilamycin. Very little was parent avilamycin. In fat, the residue appeared to be RA which had entered normal metabolic pathways and had been incorporated into fatty acids.

Comparison of the chromatograms from swine and rat livers suggests that the same metabolic profile is produced by both species. Therefore, the rats used for toxicology testing of avilamycin have been exposed to the same metabolites through autoexposure as were found in swine tissues.

ACKNOWLEDGMENT

We thank D. E. Ruggles for technical assistance and G. E. Babbitt for spectrometic analyses.

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Received for review July 19, 1990. Accepted October 3, 1990.

Registry No. Avilamycin, 11051-71-1; flambic acid, 55956-49-5; methyl flambate, 55728-97-7; flambalactone, 55729-17-4; dichloroisoeverninic acid, 4101-80-8.